MED
T113
+Y12
6583

Postnatal Development of Olfactory Receptor Cell Axonal Arbors

Jason R. Klenoff

Yale University

1998

YALE
UNIVERSITY

CUSHING/WHITNEY
MEDICAL LIBRARY

Permission to photocopy or microfilm processing of this thesis for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.

Signature of Author

3/24/98

Date

Digitized by the Internet Archive
in 2017 with funding from
Arcadia Fund

https://archive.org/details/postnataldevelop00klen

Postnatal Development of Olfactory Receptor Cell Axonal Arbors

A Thesis Submitted to the

Yale University School of Medicine

In Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

by

Jason R. Klenoff

A.B. Harvard University 1994

Yale University

1998

Med Lib
T113
+Y12
6583

YALE MEDICAL LIBRARY

AUG 1 8 1998

ABSTRACT

POSTNATAL DEVELOPMENT OF OLFACTORY RECEPTOR CELL AXONAL ARBORS

Jason R. Klenoff. Department of Neurosurgery and Section of Neurobiology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520-8082

The mechanisms that subserve the distribution of the terminal arbors of olfactory receptor cell axons remain unknown. Elsewhere in the central nervous system, a common theme is early axonal exuberance followed by activity-dependent pruning to achieve the mature distribution. This led to the hypothesis that the orderly morphology of afferent axons in the olfactory glomerulus may follow a similar developmental scheme of exuberance followed by pruning. To test this hypothesis we studied morphological features of olfactory receptor neuron (ORN) axonal arbors on postnatal days 0, 3, 6, 9, 12, and 21. The olfactory bulbs from Sprague-Dawley rats were processed using a Golgi technique that impregnated ORN axons. Axons from each age group were reconstructed by using camera-lucida at 100X, oil immersion, and morphometrically characterized. In the adult, the percent glomerular area occupied by a single ORN axon was 14% while the mean length of branches was 169.67μm, the sum of branches and varicosities was 27, and the distance to first branch point in glomeruli was 21.98μm. The values from the younger age groups were not statistically different from those in the adult. Because there was no evidence of early exuberance, our data suggest that ORN axons must innervate single glomeruli and arborize in a highly specific manner to achieve the adult pattern. Because our data suggest that ORN axons do not follow the hypothesized scheme, it is plausible to suggest that as ORN axons innervate a glomerulus during development they arborize to their adult levels but not beyond. This argues strongly that specific cell surface and trophic factors are used by the ORN axon to guide glomerular targeting and innervation.

ACKNOWLEDGMENTS

The author expresses his deepest gratitude to Dr. Charles A. Greer. Without his generous guidance and support this thesis would not have been possible, and would certainly not have been as enjoyable an experience as it was.

There can not be enough "thank you"s given to Ms. Kara Salvagno and Ms. Christine Kaliszewski for technical assistance and Dr. Karl Kafitz for valuable discussions.

Grant Sponsor: NIH; Grant Numbers: DC00210 and NS10174 to CAG. JRK was supported in part by an Alpha Omega Alpha Student Research Fellowship.

TABLE OF CONTENTS

TABLE OF FIGURES

INTRODUCTION

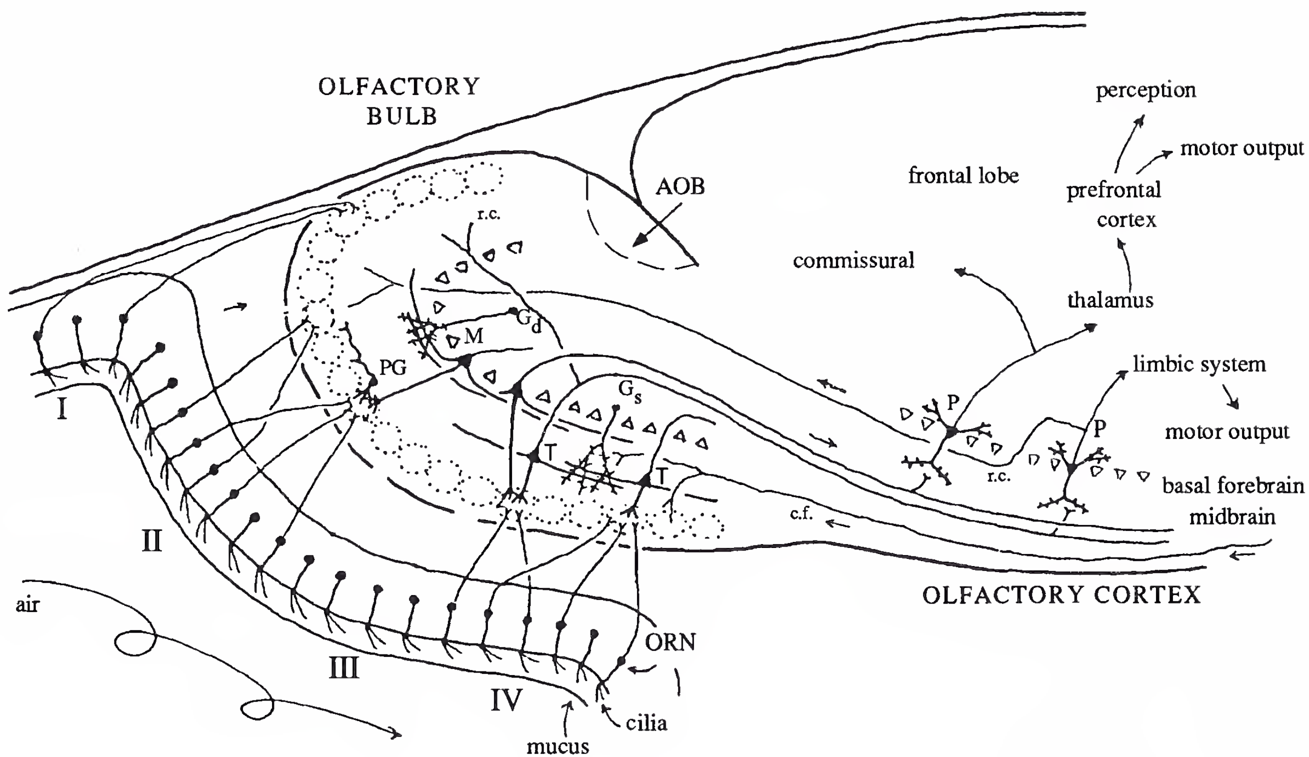
Olfactory Epithelium

The olfactory system is a complex neural network with projections from the olfactory epithelium to the olfactory bulb (OB) and from the bulb to the olfactory cortex (Fig. 1). This sensory system has been extensively reviewed in recent literature (Shepherd and Greer, 1998; Serby et al., 1992; Greer, 1991; Laing et al., 1991). The olfactory system is initially stimulated when air carrying odorants courses through the nasal cavity and comes in contact with the olfactory epithelium. The surface of the nasal cavity is largely covered by a pseudostratified ciliated respiratory epithelium. In addition, though, one can find olfactory epithelium. In humans the olfactory epithelium is located to a large degree on the dorsal surface of the nasal cavity. There one may find a certain degree of mixing of olfactory and respiratory epithelium. This contrasts with most vertebrates in which the division between respiratory and olfactory epithelium is a sharp one.

The olfactory epithelium contains four major cell types: supporting cells, basal cells, microvillar cells, and olfactory receptor cells. The supporting cells have an apical pole which sends out large numbers of microvilli. At the basal pole these cells taper and attach at the basal lamina by foot-like processes. Supporting cells make contact with one another as well as with olfactory receptor cells. Towards the epithelial surface areas of contact between supporting cells and olfactory receptor cells tend to be defined by junctional complexes. Supporting cells have been suggested to play multiple roles in addition to support including secretion of various molecules as well as possible inactivation of odorants (Getchell et al., 1988; Shepherd, 1989).

Basal cells are the stem cells which give rise to replacement olfactory receptor cells throughout the life of an individual. These cells are located in the lower epithelium near the lamina propria. They are approximately 6μm in diameter.

Figure 1: Schematic picturing the olfactory pathway. Olfactory receptor neurons travel from the olfactory epithelium to arborize within the olfactory bulb. The olfactory bulb contains neurons which then project to the olfactory cortex. The olfactory epithelium consists of four broad zones of olfactory receptor neurons which project to specific glomerulii. Abbreviations: OSN, olfactory sensory neuron; PG, periglomerular cell; M, mitral cell; T, tufted cell; Gs, superficial granule cell; Gd, deep granule cell; r.c., recurrent axon collateral; c.f., centrifugal fiber; P, pyramidal cell (modified with permission from Shepherd and Greer, 1998).


OLFACTORY
BULB
AOB
frontal lobe
perception
motor output
prefrontal
cortex
commissural
thalamus
limbic system
motor output
basal forebrain
midbrain
r.c.
G_d
M
PG
G_s
T
T
P
P
r.c.
c.f.
I
II
III
IV
air
ORN
mucus
cilia
OLFACTORY CORTEX

Also found within the olfactory epithelium is the microvillar cell. These are flask shaped cells whose role has yet to be well elucidated.

The last component of the olfactory epithelium is the olfactory receptor cell. It is a slender bipolar neuron. Its mature cell body measures about 5-8μm in diameter. A dendrite extends from the apical aspect of the cell to the mucosal surface where it ends in an expanded vessicle. From this vessicle a number of cilia arise which extend along the sensory surface of the olfactory epithlium. It has been suggested that these cilia are the site of olfactory receptor molecules (Menco and Farbman, 1985a; Menco and Farbman 1985b). The axon of the olfactory neuron originates from the basal aspect of the receptor cell. The axon is approximately .2μm in diameter and remains unmyelinated.

Olfactory Map Formation

Detection of an odorant is initiated with the binding of an odor ligand to an odor receptor contained within the membrane of an olfactory receptor neuron (ORN). ORNs are believed to express only one, or a small number of odor receptors (Chess et al., 1994) and, depending on the specific receptor expressed, are segregated into one of four broad zones within the nasal epithelium (Ressler et al., 1993; Vassar et al., 1993; Strotmann et al., 1995). Upon leaving the epithelium ORN axons coalesce to form tightly packed fascicles that penetrate the cribiform plate. When the ORN axons reach the OB they reorganize within the olfactory nerve layer (ONL) into glomerular-specific fascicles (Greer et al., 1994, 1995; Mombaerts et al., 1996). The ORNs expressing a specific odor receptor mRNA innervate only two, or at most a small number of OB glomeruli (Vassar et al., 1994; Ressler et al., 1994; Mombaerts et al., 1996). As the ORN axons reorganize and target specific glomeruli within the OB a topographically defined map of odor receptor specificity emerges. It is this map that is generally believed to underlie the results obtained with functional measures of odor processing in the OB such as those initially described with 2-deoxyglucose (Sharp et al., 1975; Stewart et al., 1979; Greer et

al., 1982) and more recently with analyses of *c-fos* (Onoda, 1992; Sallaz and Jourdan, 1993; Guthrie et al., 1993).

The innervation of a glomerulus by an ORN is central to establishing functionally defined glomerular maps. In the adult, individual ORNs innervate only one glomerulus (Golgi, 1875; Cajal, 1911; Halasz and Greer, 1993). Interestingly, single axons in the adult do not broadly innervate the glomerulus but appear restricted to smaller compartments (Halasz and Greer, 1993). This observation has contributed to the suggestion that glomeruli may include subcompartments that can be distinguished, in part, by the subpopulation of axons terminating within each compartment (Land et al., 1971; Greer et al., 1995; Treloar et al., 1996).

The initial development and arborization of the ORN axons in the OB is not yet well understood. Several lines of evidence indicate that early in development (i.e., embryonic day 16 – postnatal day 1) several individual axons may bypass the glomerular layer (GL) when innervating the OB and extend further to the ependymal core where they may contact putative projection neurons (Monti-Graziadei et al., 1980; Gong et al., 1995). As suggested, these axons may contribute to the initiation of development in the OB and may also serve as pioneer fibers for axons developing somewhat later. Santacana et al. (1992) suggested an exuberant or nonspecific development of the ORN projections extending into the early postnatal period. Their studies of the olfactory nerve failed to show evidence of clear glomerular borders, leading them to conclude that there was a lack of glomerular targeting or specificity early in development. Such an observation could be consistent with the notion of activity-dependent pruning of axonal arbors, as has been described for the visual system.

VISUAL SYSTEM

The visual system demonstrates two major classes of developmental schemes: those which are activity dependent and those which are activity independent, both of

which have been well reviewed within the recent literature (Garrity and Zipursky, 1995; Goodman and Shatz, 1993; Shatz, 1990). The initial projections of retinal ganglion cells to the optic tectum seem to be activity independent. Thus, the retinal ganglion cell growth cone can travel from the retina to the optic tectum through the utilization of the cell adhesion molecules. These molecules allow interactions between the retinal ganglion cell and its environment as well as other cells. Both attractive and repulsive forces act to guide the growing axon. Utilizing activity independent mechanisms the retinal ganglion cell is able to set up very broad, imprecise axonal arbors within the optic tectum. A coarse topographic map is set up in this manner. This map is initiated in the retina where retinal ganglion cells lie adjacent to one another and form a sort of two-dimensional array of cells. Retinal ganglion cell axons and their projections within the optic tectum remain in a similar configuration preserving this topographic map. Thus, retinal ganglion cells which are adjacent in the retina will have projections which lie close to one another within the optic tectum.

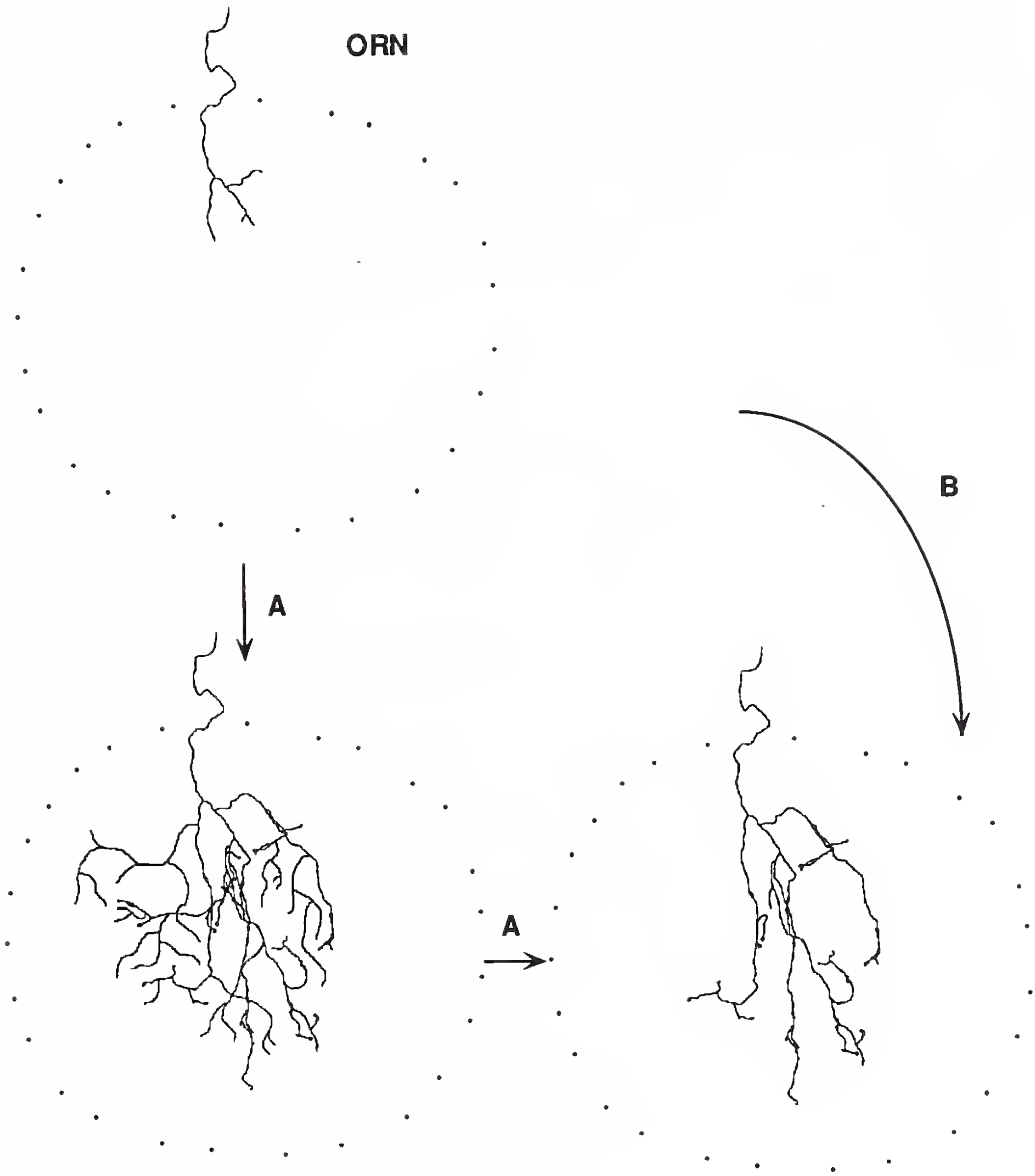
While this topographic map may be grossly created through activity independent mechanisms, it is through activity dependent mechanisms that the true refinement of this map occurs. The final map should be one in which retinal ganglion cells adjacent to one another within the retina project precisely to the optic tectum where adjacent orderly axonal arbors are set up. This configuration is set up by means of correlating electrical activity of retinal ganglion cells. The retinal ganglion cells which are side by side within the retina should be triggered in a similar manner by a given visual stimulus. Thus retinal ganglion cell projections which are simultaneously activated most likely originate from nearby areas within the retina. The visual system utilizes this fact by strengthening axonal projections whose activity correlates with its surroundings. Conversely projections whose activity does not correlate well with their surroundings are allowed to degenerate. As development proceeds exuberant projections are pruned back to the adult pattern. In this manner the retinal topographic map is conveyed to the axonal projections

within the tectum by way of activity dependent development. It seems as though a key player in these developmental mechanisms is the NMDA receptor which could allow for recognition of correlated activity patterns. In any event, blocking functional activity prevents the emergence of the adult pattern, demonstrating clearly that in the visual system, the maturation of the final map is an activity-dependent process; correlated activity among subsets of terminals leads to stabilization (Constantine-Paton et al., 1990).

HYPOTHESIS

In the olfactory system, the ORNs converging on specific glomeruli are not spatially contiguous within the epithelium. Nevertheless, an activity-dependent mechanism, such as occurs in the visual system, could contribute to the final adult pattern in which ORN axons are restricted to a single glomerulus. Alternatively, pathfinding and the selection of postsynaptic targets by ORN axons could exhibit a high degree of specificity such that the adult pattern emerges seamlessly over the course of development. Such an observation might suggest that activity-dependent tuning or sharpening of axonal arbors does not contribute to the establishment of olfactory receptor specific glomerular maps in the olfactory system (Fig. 2). To explore these alternatives more fully we have reconstructed with camera-lucida Golgi impregnated ORN axons and quantified their terminal arbors over the course of postnatal development. The data reveal that ORN axons follow a linear course of development beginning as a simple unbranched process that matures to the adult pattern of arborization without any evidence of exuberant or non-specific inter- or intraglomerular targeting.

Figure 2: This experiment tested two alternative hypotheses of ORN axon development. (A) shows the possibility of exuberant development of the axonal arbor followed by pruning via activity dependent mechanisms. (B) shows the alternative of precise initial innervation of the olfactory glomerulus without excessive branching.


ORN
A
B
A

MATERIALS AND METHODS

TISSUE PROCESSING

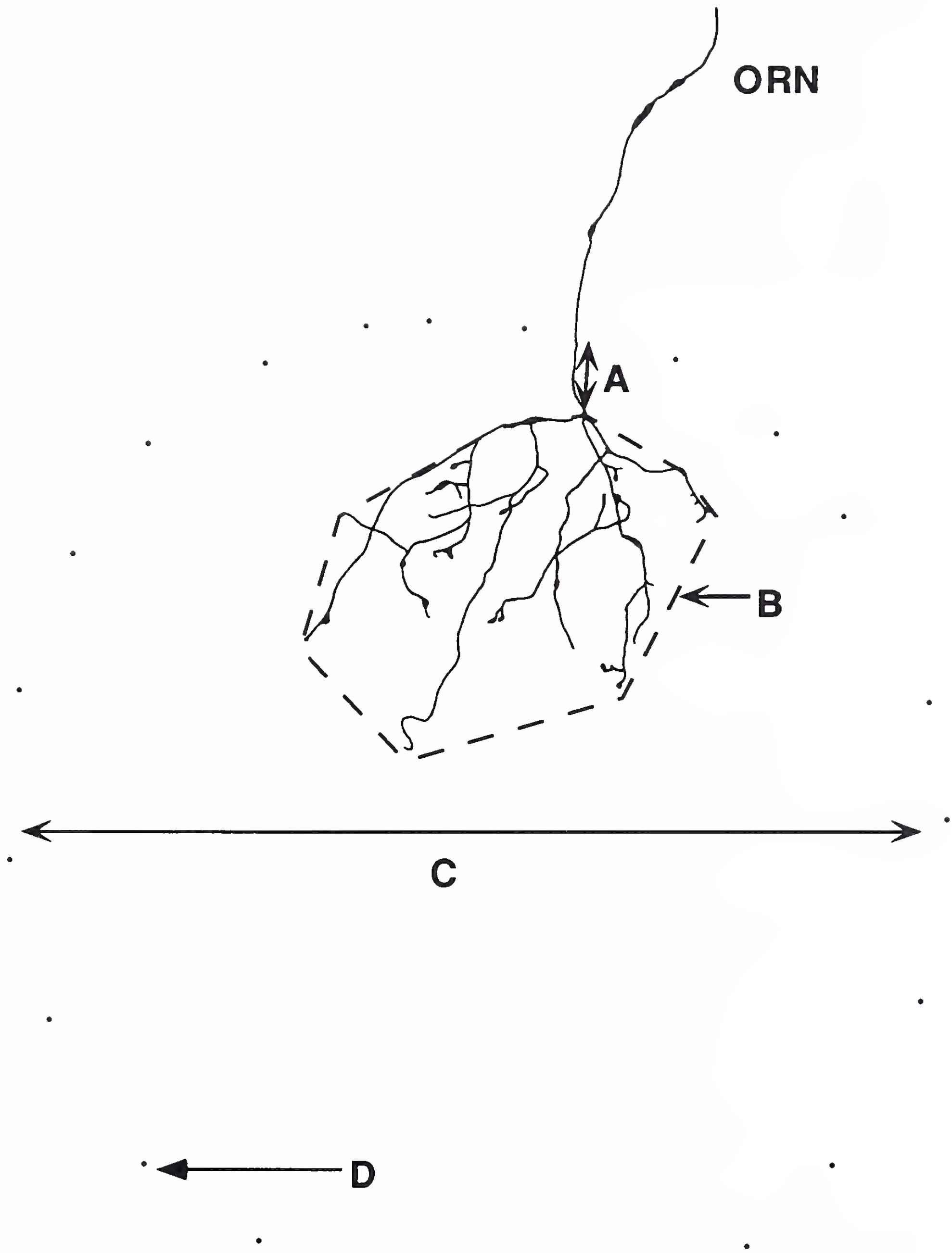
Sprague Dawley rats were studied at ages 0 (n = 5), 3 (n = 3), 6 (n = 6), 9 (n = 3), 12-13 (n = 6), and 21 (n = 5) days postnatal (DPN). All protocols were reviewed and approved by the Yale Animal Care and Use Committee (YACUC # 07161). At 9, 12-13, 21 DPN rats were anesthetized with an intraperitoneal injection of 70mg/kg Nembutal and perfused through the heart with 0.9% saline followed by 1.25% paraformaldehyde and 1.25% gluteraldehyde in 0.1M phosphate buffer. Following the perfusion the OBs were removed and placed in fresh fixative overnight at $4^{O}C$. At 0, 3, and 6 DPN rats were anesthetized by immersion in an ice bath and then decapitated. The brain was left in situ and immersed in the fixative solution overnight. The OBs were subsequently processed with a Golgi technique that allowed resolution of individual ORN axons (Halasz and Greer, 1993; Cameron et al., 1991). Briefly, following fixation, the brain was incubated for 24 hours in a dichromium osmium solution (70ml 4% dichromate in 0.1M phosphate buffer added to 10ml of 2% osmium) followed by 24 hours in 0.75% silver nitrate in distilled H_2O. Subsequently, the tissue was placed in increasing concentrations of glycerol (2.5%, 20%, 40%, 60%, 80%, 100%) for 5 minutes each. The brains were then embedded in agar and 80 m slices were cut from the OBs on a Vibratome. After a 30 minutes exposure to bright light the slices were sequentially placed in decreasing concentrations of glycerol (80%, 60%, 40%, 20%, 2.5%). OB slices were then placed in a gold solution (3.5ml 1% gold chloride, 48.75ml distilled H_2O, and 1.25ml 100% glycerol) for 35 minutes while being kept at $4^{O}C$ in the dark. The tissue was rinsed in distilled H_2O and placed in a 0.2% oxalic acid solution for 4 minutes at $4^{O}C$ and again in distilled H_2O and then placed at room temperature in a 1% sodium thiosulfate solution 2 X 30 minutes Slices were rinsed in distilled H_2O then dehydrated.

Dehydration began with a rinse of 70% ETOH for 10 minutes Subsequent dehydration rinses were: 1% uranyl acetate in 70% ETOH for 1 hr., 90% ETOH for 10 minutes, 100% ETOH 2 X 15 minutes, propylene oxide 2 X 5 minutes, and 1:1 mixture of propylene oxide and EPON for 1 hr. Slices were then placed in Epon over night. The next day slices were placed in fresh EPON for 2 hours then embedded in Epon on slides coated with Quick Release Agent (Hobby Time, Bodensee, Germany) and polymerized at 65^{o}C for 24 hours.

Axonal Reconstruction

Light microscopy was employed to examine labeled axons. Using 100X oil immersion (total magnification = X1650), camera-lucida reconstructions were made of ORN axons and the limits of the glomeruli they innervated. Only those axons completely stained and with axonal arbors limited to a single section were studied. Of the axons analyzed, a minimum of 20 from each age group were reconstructed with camera-lucida at 100X oil immersion. The camera-lucida drawings were then digitized by scanning them into a Power Macintosh 7100 with a UMAX UC1260 scanner. Using NIH Image 1.58, the axonal arbors were evaluated on the basis of number of branches, number of varicosities, total length of branches, distance to first branch, and total branch area occupied (Fig. 3). Number of branches and number of varicosities were assessed as the total number after the axon had entered the glomerulus. Length to first branch (μm) was measured from the point where the axon entered the glomerulus to the first branch point within the glomerulus. The total length of branches (μm) was measured as the summed length of all branches from the first branch point within the glomerulus onwards. Total branch area (μm^2) was measured by calculating the area of maximal extension of an arbor. This was determined by constructing a polygon whose edges connected the furthest extensions of branches within the arbor. The area of this polygon was defined as the total branch area (μm^2). The parameters described above allowed evaluation of

Figure 3: Camera lucida drawing illustrating a typical ORN axon and the morphometric parameters that were measured. (A) distance to the first branch point; (B) dashed line depicts the outer limits of the area of arborization; (C) diameter of the glomerulus; (D) dotted line depicts the outer limits of the glomerular boundary. The percent of glomerular area occupied by an ORN axon is equivalent to the area contained within B divided by the area contained within D.


ORN
A
B
C
D

axonal arbor size and density during the course of postnatal development. Also evaluated were the glomerular area (μm^2) and glomerular diameter (Fig. 3). Glomerular area (μm^2) was determined by the outer boundaries of the glomerulus, while glomerular diameter was calculated as the average of the major and minor axes of the glomerular boundary. Beginning at P3 glomerular boundaries were determined on the basis of surrounding juxtaglomerular cells. At P0, glomerular boundaries were only roughly established by mapping the distribution of the axonal neuropil under high contrast microscopy (10 - 20X objectives). Quantitative characterization of the glomeruli was not performed at P0. All variables were statistically evaluated with a MANOVA.

Axons were further evaluated in the nerve layer using 100X oil immersion. Stained axons were followed within the nerve layer and the number of branch points and length of axons and branches were recorded. A minimum of 3000µm of stained axon was evaluated for each of the six age groups studied.

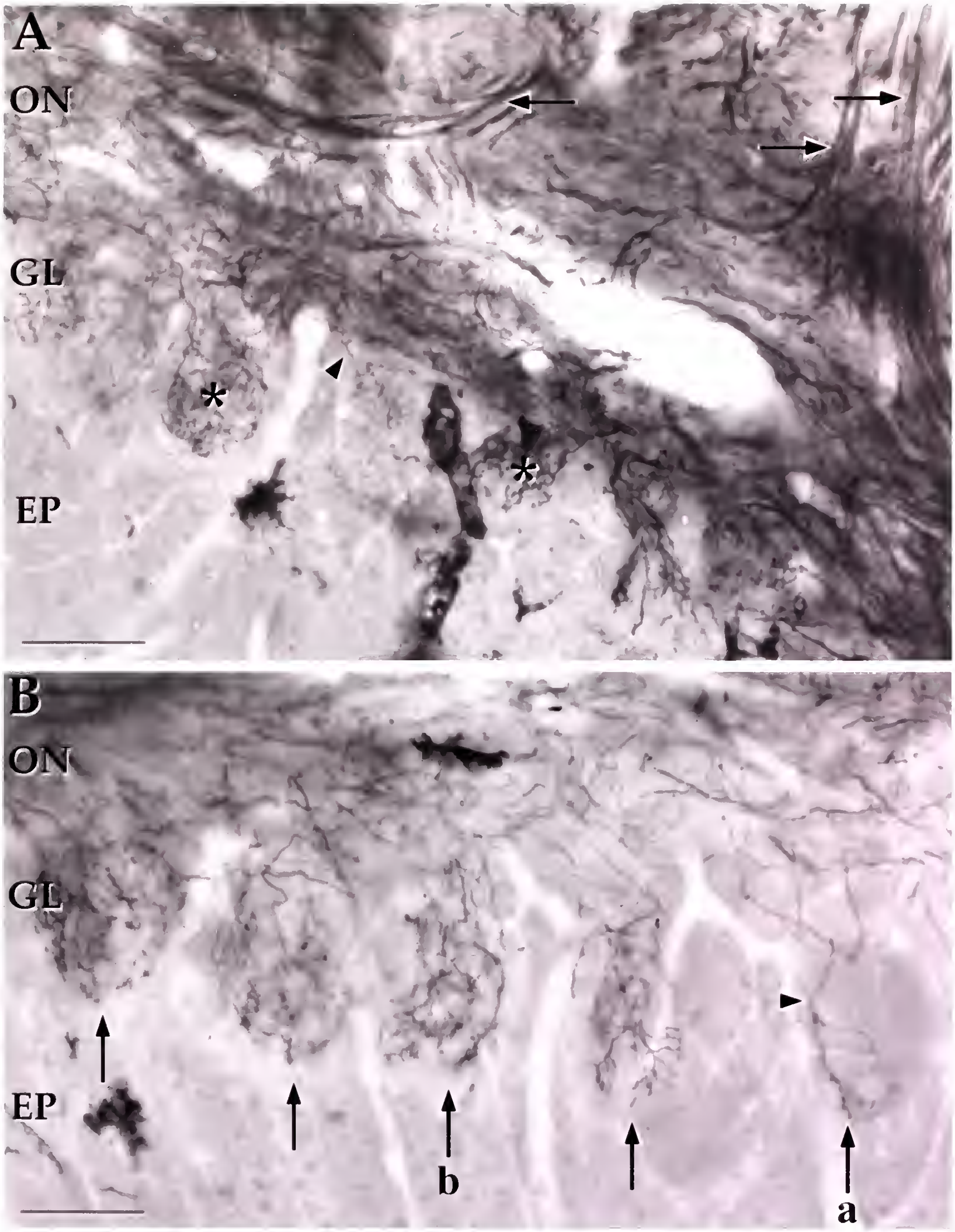
Photographic images were obtained with Ektachrome 160 on an Olympus BH2 microscope by using 4X - 100X, oil immersion, objectives. Selected images were scanned into a Power Macintosh 7100 by using a Microtek Slide Scanner. Adobe Photoshop 3.0 was used to composite individual images into the final figures (Figs. 2 and 3). Adobe Photoshop filter sets were not employed although brightness was equalized across each of the images included in a figure.

RESULTS

Axonal Staining

Effective staining was accomplished throughout all of the age groups with the Golgi methods employed. As shown in the adult in Fig. 4A, within the ONL well defined fascicles of ORN axons were easily identified (arrows). Fascicles coursed through the ONL in fairly nontortuous routes. Axons separated from and then joined new fascicles as

Figure 4: (A) Golgi stain showing the olfactory nerve layer (ON), the glomerular layer (GL), and the external plexiform layer (EP). ORN axons are seen in isolation and as components of larger fascicles (i.e., arrows) within the ON. As fascicles approach and innervate glomeruli (i.e., asterisk) the axons separate and arborize within the glomerular compartment (arrowhead). Scale bar = 100μm. (B) Golgi stain showing glomeruli (arrows) containing variable amounts of stained ORN axonal arbors. Some glomeruli (i.e., a) contained single axons whose arbors could be easily followed (arrowhead), while in more densely stained glomeruli (i.e., b) it was not possible to follow single axons. Scale bar = 50μm.


A
ON
GL
EP
*
*
B
ON
GL
EP
b
a

they approached target glomeruli. Within the glomerulus, axons generally arborized in a localized or restricted compartment, as we have previously reported for the adult (Halasz and Greer, 1993). In addition to complex fascicles of axons (Fig. 4A, arrows), individual ORN axons could be followed from the ONL to their arborization within the glomerular neuropil (Fig. 4A,B, arrowheads). In each OB slice individual glomeruli contained variable numbers of stained ORN axons (Fig. 4B). In the more densely stained glomeruli it was not possible to effectively follow individual axons. However, these instances did allow evaluation of broader characteristics of ORN innervation of glomeruli. On the other hand, those glomeruli that contained only a limited number of stained axons allowed a detailed assessment of morphological features and patterns of arborization for single axons. At high magnification it was possible to delineate the specific morphology of individual axons, including varicosities and terminal boutons.

Axonal Morphology

Fascicles of axons could be delineated coursing through the ONL in all age groups. Axons were found to rarely branch within the nerve layer with a frequency of <1/3,000μm across the age groups (Table 1). Moreover, in the few instances identified, the branches found were ≤ 10μm long (Table 1). Upon leaving the nerve layer fascicles of varying sizes were seen penetrating and arborizing within glomeruli. At the earliest age at which glomerular borders were clearly delineated, 3 DPN, it was evident that single axons obeyed a general rule of being restricted to single glomeruli. In addition to the rarity of branching within the nerve layer, of all the ORN axons studied, including those not reconstructed for final analysis, only three (<0.1%) had arbors which contained branches in more than one glomerulus (i.e., Fig. 5). These axons were not restricted to the younger age groups. At 0 DPN, though glomerular boundaries were more difficult to establish, branching in the nerve layer remained rare, and there was no evidence to suggest that individual axons did not obey the rule of 1 axon : 1 glomerulus.

	0 DPN	3 DPN	6 DPN	9 DPN	12 DPN	21 DPN
Number of Axons	38	35	33	40	29	31
Total Length of Axons (µm)	3070	3030	3015	3045	3045	3045
Number of Branches	1	1	0	0	1	0
Branch Length (µm)	3	5	-	-	10	-

Table 1: The frequency and length (µm) of secondary axonal branches in the olfactory nerve layer of the rat olfactory bulb at 0, 3, 6, 9, 12 and 21 days postnatal (DPN).

Figure 5: (A) Golgi stain showing an unusual ORN axon (arrows) from a 12 DPN rat which contains branches in two glomeruli. Both the olfactory nerve layer (ON) and the glomerular layer (GL) can be seen. Scale bar = 50μm. (B) Camera lucida reconstruction of the ORN axon shown in (A). The arrows in (A) and (B) identify corresponding points. Dots denote the glomerular boundaries. Scale bar = 20μm.

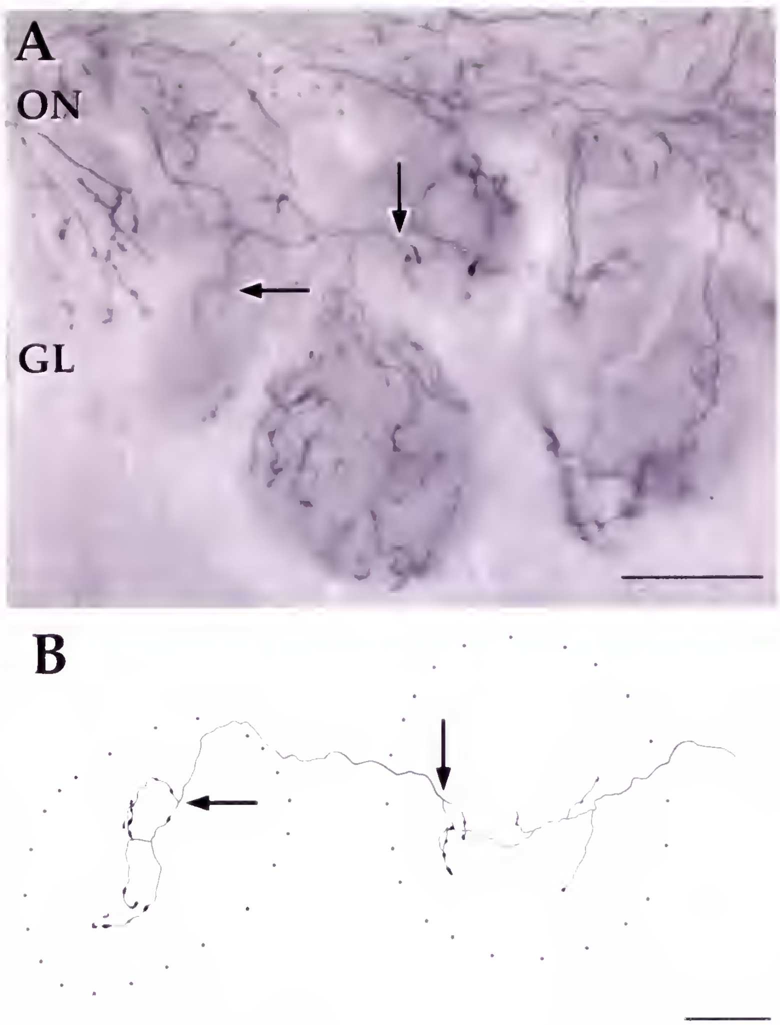

A
ON
GL
B

Fig. 6 shows examples of Golgi-impregnated ORN axons from all of the age groups studied. The fundamental characteristics of ORN axons did not change across development. Thus, axonal diameter, approximately 0.2μm, was equivalent across the age groups. The dimensions of varicosities (0.3-1.2μm) and terminal boutons (0.3-1.0μm) were also similar throughout development. For example, at 0 DPN (Fig. 6A) an axon is shown that contains two varicosities which are closely spaced (arrowheads). It also shows the fairly straight course which tended to be followed by axons before reaching their respective glomeruli. In an example from 3 DPN (Fig. 6B) one can see how the ORN axons tended to make abrupt turns (arrowhead) upon innervating a glomerulus and a subsequent terminal field. One can also see that branching occurs in this example only after the axon has traveled approximately 20μm into the glomerulus. Axonal branching was generally not evident in any of the age groups until an ORN had entered the glomerular neuropil. In the example from 6 DPN (Fig. 6C) the axon takes a particularly long straight route before beginning to arborize (arrow) within the glomerulus. In the example from 9 DPN (Fig. 6D) a branch point (arrow) and adjacent varicosity (arrowhead) within the glomerular neuropil are indicated. At 12 DPN (Fig. 6E) an axon is shown undergoing abrupt changes in direction (arrowheads). Despite these turns axons tended to follow a fairly straight course through the ONL. Finally in an example from 21 DPN (Fig. 6F) an axon is shown at its initial point of arborization within a glomerulus. A specific branch point is marked (arrow). No clear varicosities or terminal boutons are apparent within this field of view.

AXONAL GLOMERULAR ARBORS

<u>0 DPN</u>

In 0 DPN rats the ORN axons studied contained all of the morphological components of older rat ORNs, including varicosities and terminal boutons (Fig. 7A-C, arrowheads). However, these features tended to be encountered less frequently than in

Figure 6: Golgi-impregnated axons from each of the age groups studied. (A) ORN axon from a 0 DPN rat. Note the two closely spaced varicosities (arrowheads). (B) ORN axon from a 3 DPN rat. Branching does not occur until the axon has traveled ~20 μm into the glomerulus. Axons often made abrupt turns (i.e., arrowhead) as they traveled toward their terminal field. (C) ORN axon from a 6 DPN rat. Note the long course the axon follows prior to arborizing (i.e., arrow) within the glomerulus. (D) ORN axon from a 9 DPN rat. A branch point (arrow) and adjacent varicosity (arrowhead) are indicated. (E) ORN axon from a 12 DPN rat. Axons could undergo abrupt changes in direction (arrowheads). (F) ORN axon from a 21 DPN rat. At the site of initial arborization within the glomerulus a branch point (arrow) is seen. Scale bars = 10μm.

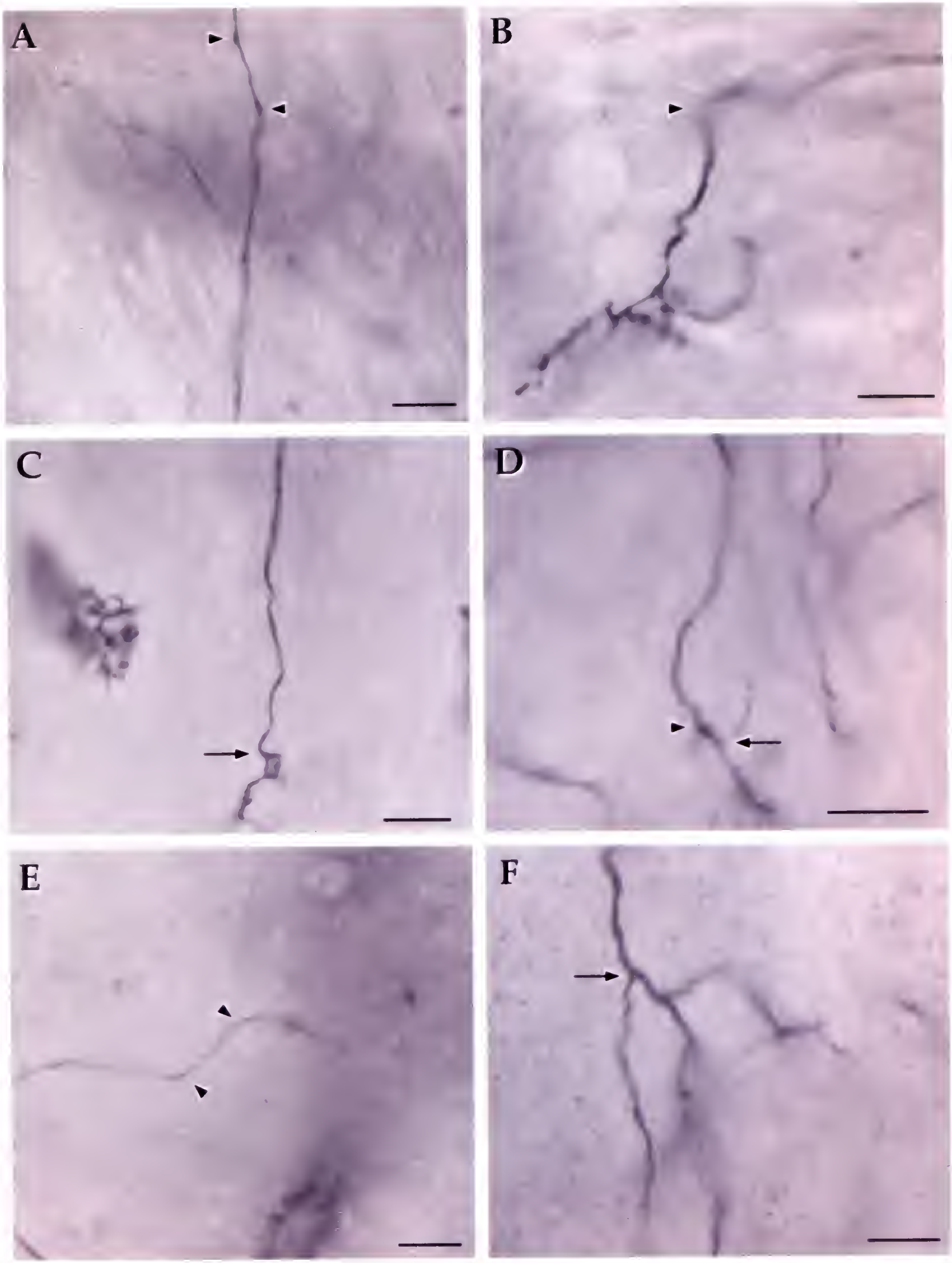
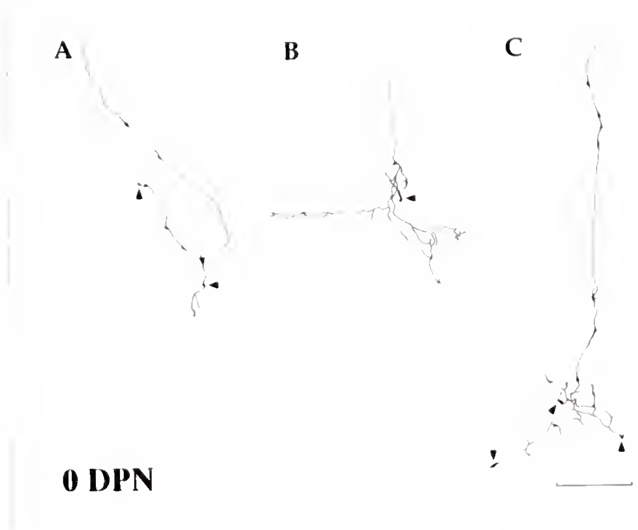

A
B
C
D
E
F

Figure 7: Camera-lucida reconstructed ORN axons from 0 DPN rats. In (A), (B) and (C) the axons all formed a comparatively simple arbor although varicosities and terminal boutons (i.e., arrowheads) were routinely encountered. Scale bar = 20μm.

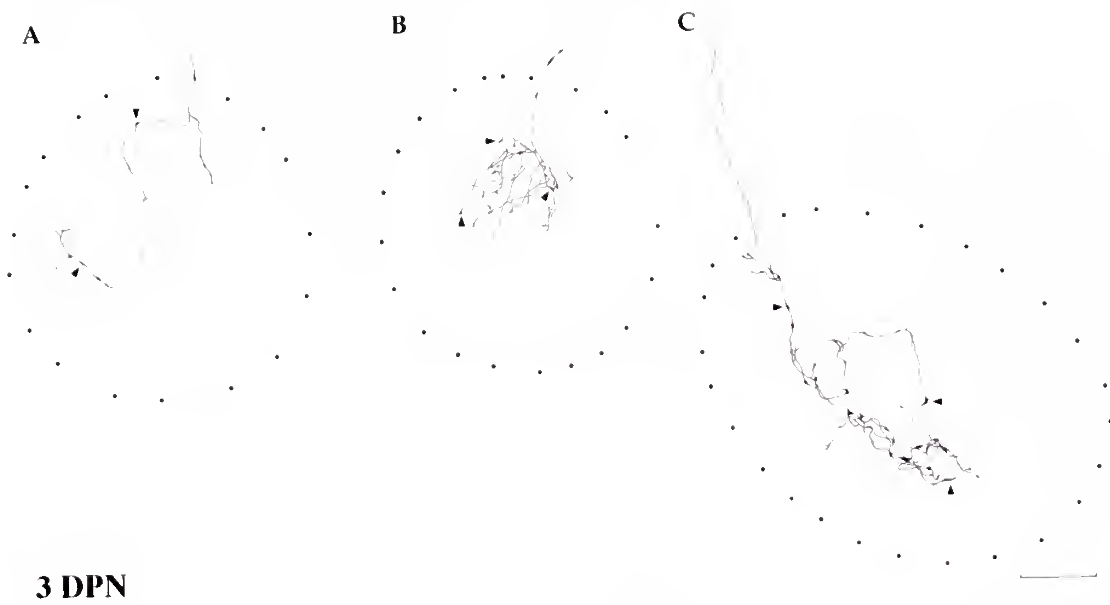

A
B
C
0 DPN

axons from older individuals (vide infra). Also of note is that the branching pattern of the axons tended to be simpler with less dense arbors than axons from older rats. In Fig. 7A is an axonal arbor with only four branches. The axon makes an abrupt turn before beginning to branch. Fig. 7B shows a more complex arbor, however, many of the branches are short. Note, as in 7A that there is a scarcity of varicosities. Fig. 7C has somewhat more varicosities. This axon travels without turning for a significant distance before beginning to branch. It also has two small branches which occur before the axon reaches its main terminal field. In Fig. 7A-C glomerular boundaries are not drawn due to the absence of the juxtaglomerular cells we used to define glomerular boundaries in the older animals. At P0, the glomerular boundaries were established under high contrast microscopy and quantitative characterization was not attempted.

<u>3 DPN</u>

In examining camera-lucida reconstructions of ORN axons from 3 DPN rats (Fig. 8A-C) it was possible to see, as in other age groups, that a variety of branching patterns were present. ORN axons often had very simple branching patterns and a paucity of varicosities and terminal boutons (arrowheads in Fig. 8A). In this regard the arbor shown in Fig. 8A is similar to those seen more frequently in the axons from 0 DPN rats. Note that after the first branch point one of the branches travels a long distance before branching again. The 3 DPN rats also had axons with much more complex arbors (e.g. Fig. 8B,C). These examples show a higher degree of branch density and numerous varicosities and terminal boutons. The axon shown in Fig. 8B exhibits extensive branching within a small area of the glomerulus. This pattern of branching was evident in many ORN axons and is suggestive of subcompartmentalization within the glomerulus, as was previously described in the adult (Halasz and Greer, 1993). Note that in this example the arbor is apparently in the central area of the glomerulus. Fig. 8C is a complex arbor which is not as compact as 8B. It has a few small branches before the axon enters its major terminal field.

Figure 8: Camera-lucida reconstructed ORN axons from 3 DPN rats. The axon shown in (A) is relatively free of complex branching and exhibits few varicosities or terminal enlargements (arrowheads). In contrast, the examples shown in (B) and (C) are more "adult-like" in their subcompartmental distribution within the glomerulus and density of varicosities and terminal boutons (arrowheads). Scale bar = 20μm.


A
B
C
3 DPN

6 DPN

Camera-lucida reconstructions of axons from 6 DPN rats again demonstrate the variability of axonal branching patterns (Fig. 9A-C). Branching tended to be localized as seen in Fig. 9A,C. However, a few examples of more diffuse arbors were also evident as in Fig. 9B. Fig. 9A shows a complex localized arbor with multiple varicosities and terminal boutons (arrowheads). This reconstruction also demonstrates how axons could make abrupt turns before entering a glomerulus. Upon entering the glomerular neuropil, the axon does not arborize until traveling about 20μm. Fig. 9B shows an axon which, like that shown in Fig. 9C, arborizes immediately upon entering the glomerular neuropil. It, however, has one branch which travels along the edge of glomerulus for a long distance without arborizing. Therefore, while the arbor is not dense, the branch area appears extensive (vide infra). Fig. 9C shows a more limited arbor which is restricted to the dorsal periphery of the glomerulus. One can see the similarities between this axon and the axon from a 0 DPN rat shown in Fig. 9B. Note the presence of varicosities outside of the glomerular boundary. Varicosities, in this example and in general, are nevertheless more frequently found within the glomerular neuropil than in the ONL.

9 DPN

Fig. 10A shows a simple axon with seemingly simple characteristics. It has only three branch points (arrows) and contains a limited number of varicosities and terminal boutons. This axon stays close to the perimeter of the glomerulus. It is contrasted with the axons shown in Fig. 10B,C which are more complex with a greater degree of branching and more varicosities and terminal boutons (arrowheads). They also travel to apparently more central areas of the glomerulus prior to arborizing. In Fig. 10B, as in most examples, note that some branches contain terminal enlargements while other branches do not include well defined terminal boutons. In Fig. 10C note the presence of varicosities between the point where the axon first enters the glomerular neuropil and the

Figure 9: Camera-lucida reconstructed ORN axons from 6 DPN rats. The axon shown in (A) depicts a relatively complex arbor with multiple varicosities and terminal boutons (arrowheads). In (B) is an example of an axon whose arbor is extensive but has a lower density of branches. In (C) a simpler arbor is shown that is restricted to the dorsal periphery of the glomerulus. Scale bar = 20μm.

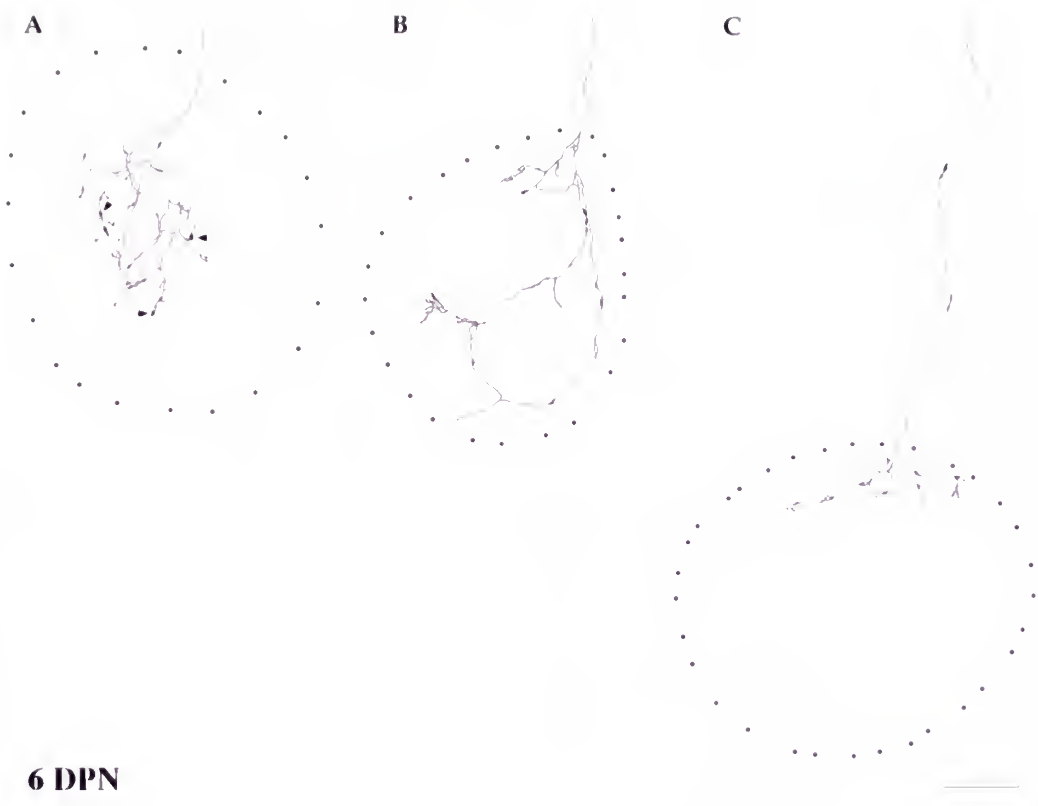
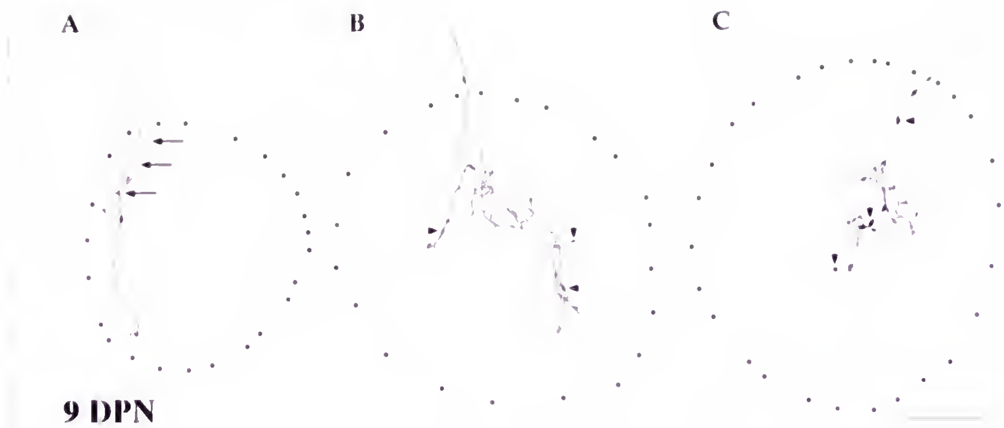

A
B
C
6 DPN

Figure 10: Camera-lucida reconstructed ORN axons from 9 DPN rats. In (A) a simple axon with only 3 branch points (arrows) is shown while in (B) and (C) more complicated axons are reconstructed with multiple varicosities and terminal boutons (arrowheads). Note in (A) that the axon stays close to the perimeter of the glomerulus while in (B) and (C) the axons penetrate toward deeper regions. Scale bar = 20μm.


A
B
C
9 DPN

point where it first branches. In looking at the other examples one can see that this is not a common occurrence. The majority of varicosities occur within the axonal arbor, after the first branch point.

12 DPN

Fig. 11A shows an extremely simple arbor with no branches. Notice, however, that morphologically the axon still contains varicosities and a terminal bouton (arrowheads). In Fig. 11B one can see two separate axons which were traveling adjacent to one another in the ONL and then innervated the same glomerulus. The axons established non-overlapping terminal fields within the glomerulus (arrows). Many examples could be found in which axons arborized within separate localized fields within the same glomerulus. Fig. 11C shows one of the more complex axonal arbors reconstructed. It is an unusual axon that appears to have an accessory terminal field (arrow) in addition to its main arbor. This axon with its many branches as well as varicosities, and terminal boutons (arrowheads) contrasts with Fig. 11A. Fig. 11B,C are examples of axons which branch prior to entering the glomerular neuropil. Branches of this type were not common and tended to be small.

21 DPN

Camera-lucida reconstructions of axons from 21 DPN rats are shown in Fig. 12A-C. Note again the variability in the breadth of the axonal arbor and in the density of varicosities and terminal boutons (arrowheads). Fig. 12A shows an axon which travels a distance before beginning to arborize. Its arbor stays mainly to one side of the glomerulus. It is not a large arbor, however, considering its size it has a large number of varicosities and terminal boutons (arrowheads). Fig. 12B shows an axon which appears to be more central within the glomerulus. It has a diffuse arbor, and fewer varicosities than are seen in 12A. One should note in Fig. 12B, and in other examples, that branch points tend to generate only two branches. Fig. 12C shows an axon which is confined to the dorsal aspect of the glomerulus. It has a simple arbor with few branches.

Figure 11: Camera-lucida reconstructed ORN axons from 12 DPN rats. In (A) an axon without branch points but exhibiting a terminal enlargement and varicosities (i.e., arrowheads) is shown. In (B) is an example in which two axons establish non-overlapping fields within the same glomerulus (arrows). In (C) is an unusual axon that appears to have an accessory terminal field (arrow) in addition to its main arbor. Note the numerous terminal boutons and varicosities (i.e., arrowheads). Scale bar = 20µm.

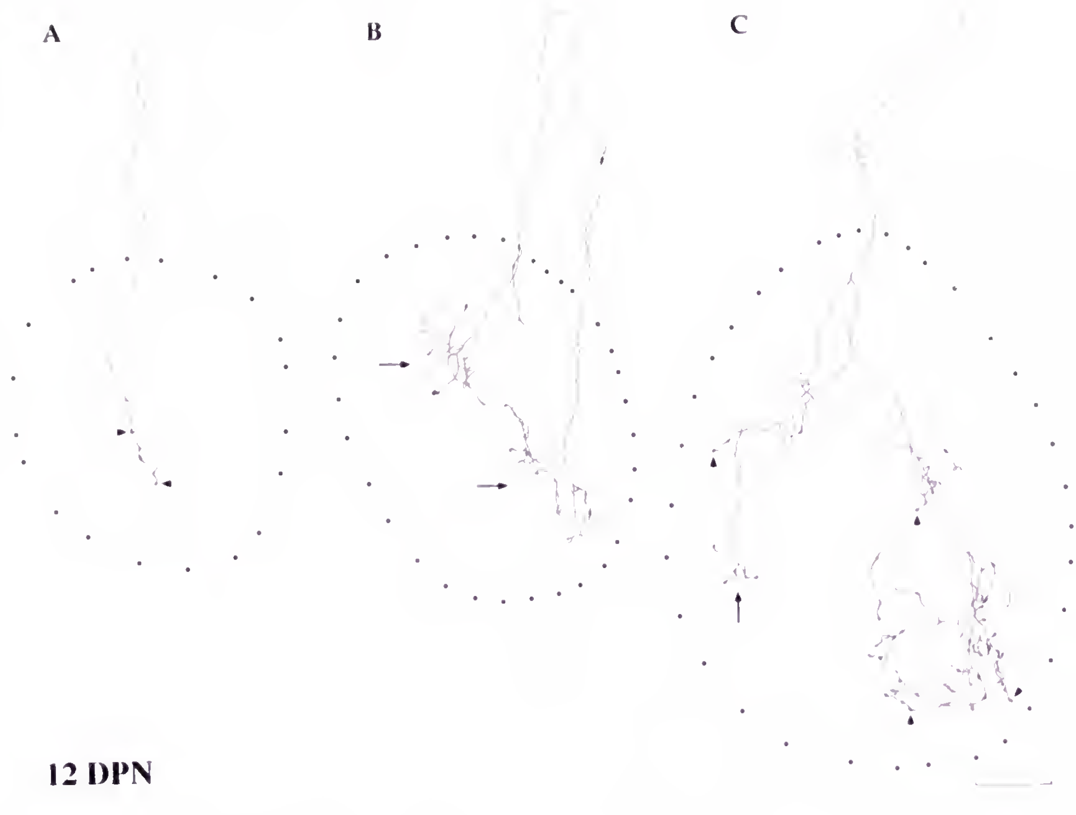
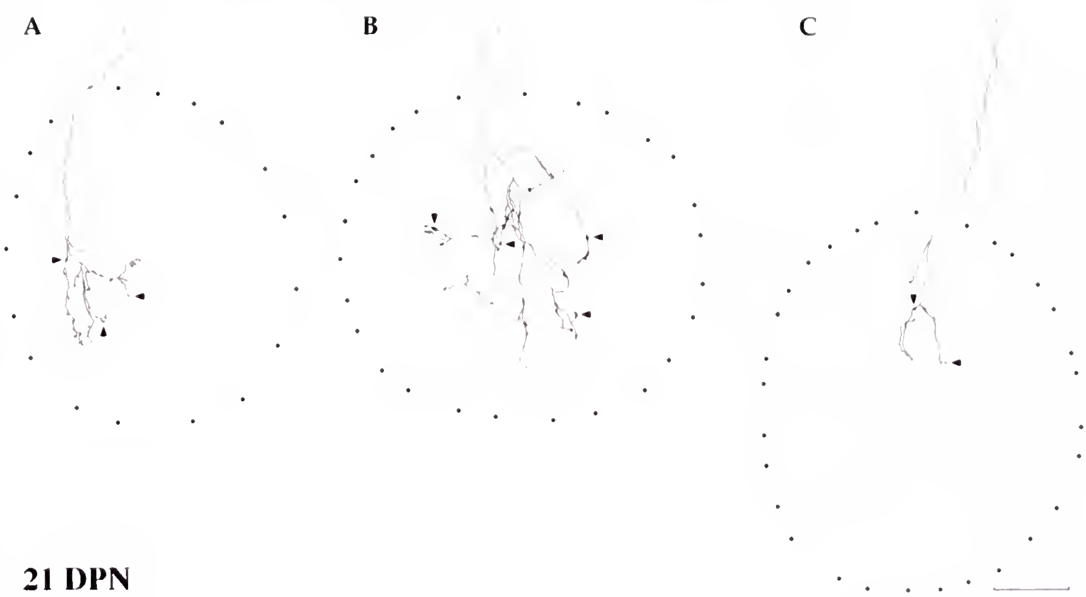

A
B
C
12 DPN

Figure 12: Camera-lucida reconstructed ORN axons from 21 DPN rats. Note in (A), (B) and (C) the variability in the breadth of the axonal arbors and in the density of varicosities/terminal boutons (i.e., arrowheads). Branching did not occur until the axon was well within the perimeter of the glomerulus. Scale bar = 20μm.


A
B
C
21 DPN

MORPHOMETRIC ANALYSIS

Once Golgi-impregnated axons from each of the age groups had been reconstructed by camera-lucida, the reconstructions were then scanned and analyzed. Using NIH Image 1.58 the axonal arbors were evaluated on the basis of number of branches, number of varicosities, total length of branches, distance to first branch, and total branch area occupied (Table 2). The total number of branch points, which is equivalent to counting the number of terminal boutons, and the total number varicosities was taken to approximate the number of synapses within an ORN axonal arbor since we had previously demonstrated that intraglomerular varicosities and terminal boutons were sites of synapse formation (Halasz and Greer, 1993). It is apparent that there was a trend towards an age dependent linear increase in variables up to approximately 6 DPN, and then a plateauing of the variables to the adult value. Within this trend there was a dip in values at 9 DPN. In none of these variables does one find a peak at one age with a subsequent decrease at future ages, as one would expect if exuberant development were present. In addition to the aforementioned variables, glomerular area was also measured and found to increase with age (Table 2). To correct for increasing glomerular area the total branch area occupied by axons and the distance to the first branch point within glomeruli were evaluated as ratios to glomerular area (Table 2). These values were fairly constant throughout age groups, including 9 DPN (Table 2). None of these variables showed a peak at one age followed by a decline as would be expected if hypertrophy were present. Variables and ratios were statistically analyzed using the MANOVA statistic. Ratios were first transformed using the arcsin (square root (p)) transformation. Using the Shapiro-Wilk W test each transformed variable was then determined to have a normal distribution, $p > .05$. The dependent variables evaluated using the MANOVA statistic were: percent of glomerular area occupied by branch area, total length of branches, number of branches and varicosities, and distance to the first branch point as a

	0 DPN	3 DPN	6 DPN	9 DPN	12 DPN	21 DPN
Number of Rats	5	3	6	3	6	5
Number of Axons Evaluated	20	20	20	20	20	20
Branch Area (μm^2)	519.69±76.61	738.93±119.18	901.94±142.11	538.61±48.66	989 16±273.51	797.78±121 86
Branch Length (μm)	114.65±9.60	173.23±20.65	184.85±21.20	136.10±10 43	194 76±40.04	169.67±18 47
#Branches + #Varicosities	18.10±1.33	28.05±3.38	29 25±3.80	26.40±2.72	29.50±5.49	26.65±2 86
1st Branch Point (μm)	-----	19,26±3.48	18.74±2.98	13.70±2.18	26.72±4.08	21.98±3 45
Glomerular Area (μm^2)	-----	4,509 68±301.00	5,336.91±179.31	4,157.70±307.23	6,519 21±426.71	5,746 41±231.19
Branch Area : Glomerular Area	-----	0.14±0.02	0.16±0.03	0.13±0.01	0.14±0.03	0.14±0.02
1st Branch Point : Glomerular Area (μm^{-1})	-----	0.0050±0.0010	0.0036±0.0006	0.0035±0.0005	0.0047±0.0009	0.0040±0.0007

Table 2: The mean (+/- S.E.M.) of the morphometric variables evaluated for the ORN axons in each age group.

ratio to glomerular area. The MANOVA for these morphometric indicators across age groups resulted in p = .14 (Wilks' Lambda). Thus, no statistically significant variation across development was found suggesting that the morphometric features measured did not demonstrate evidence of developmental exuberance.

DISCUSSION

Our results demonstrate that ORN axons innervate single glomeruli and arborize to their final adult intraglomerular pattern without evidence of either exuberance or non-specific targeting. These data suggest that targeting of glomeruli and intraglomerular processes occurs with high specificity during development. If exuberant development of the ORN axon were occurring we had predicted that the morphological variables we quantified would peak somewhere during the developmental period and subsequently decrease to the adult level. However, our data demonstrate that the number of branches, number of varicosities, total length of branches, distance to first branch, and total branch area occupied all matured to adult levels without evidence of exuberance. Nevertheless, these variables do exhibit a trend towards an age dependent linear increase up to the adult level at which point values plateaued. When comparing the morphometric axonal values to glomerular area, ratios remained constant throughout development. These results suggest that ORN axons innervate the glomerulus in a precise manner without an exuberant period during which they arborize beyond the mature limits. Therefore, these data argue for the presence of highly specific guidance and trophic markers that the ORN axons employ while selecting glomeruli and establishing a final arbor. Moreover, the data suggest that ORN axons may not utilize activity-dependent mechanisms of development as extensively as other sensory systems.

In DiI studies Santacana et al. (1992) suggested that ORN axons exhibited exuberant growth in both radial and tangential directions. The radially directed axons bypassed the glomeruli and reached the ependymal core of the OB. These have been

previously described as pioneer axons and clearly represent only a small subset of ORN axons that appear to occur largely between E13 - E16 (Monti-Graziadei et al, 1980; Gong and Shipley, 1995). Analyses of mitotic events in the proliferative zone of the embryonic OB suggest that these axons may induce an upregulation in neurogenesis and OB development (Gong and Shipley, 1995). Santacana et al. (1992) also suggest a tangential exuberance of ORN axons in the OB prior to E15 when DiI stained axons were seen spreading over the telencephalic vesicle and presumptive OB without evidence of restriction within glomerular neuropils. However, one would not expect to see glomerular borders until after E19 when the radial glia develop into juxtaglomerular astrocytes and begin to delineate the borders of glomeruli (Valverde et al, 1992). Moreover, periglomerular neurons do not begin to migrate extensively into the glomerular layer until the postnatal period (Bayer and Altman, 1975). Thus, regardless of possible hypertrophy, at E15 it would be unlikely that well formed glomerular boundaries would be detected. Rather it would be more plausible to predict that the ORN axons would appear seamlessly spreading over the telencephalic vesicle. This is especially true of the Santacana et al. (1992) DiI studies where large populations of axons were visualized rather than single processes. Thus, it seems plausible to suggest that the DiI stained axons described by Santacana et al. (1992) were not exuberant, in the conventional sense, but rather were large populations of ORN axons that were not yet segregated by clear glomerular boundaries.

In the current study embryonic development was not assessed. Nevertheless, it still seems unlikely that exuberance of ORN axonal arbors is a principle of development during the embryonic period without being manifested during the early postnatal stages while ORN production and innervation of the OB is still very robust, as is evident in the strong trend towards increasing complexity of the axonal arbor in the older versus the younger animals. This can be seen in comparing 0 DPN axonal arbors (Fig. 7) to arbors from older individuals (Fig.s 8-12). It does not seem plausible that subsets of axons

would undergo hypertrophy during embryonic development only to decrease in complexity in the early postnatal period and then once again increase to the adult level. Moreover, if activity-dependent pruning were a major determinant of ORN axonal maturation, it would seem more likely that it would take on a major role in the postnatal period when an individual was being exposed to new environmental stimuli. Thus, while this study did not specifically examine embryos, it does not seem likely that exuberance would be a predominate feature of ORN axonal development.

The evidence against exuberance in the development of the ORN axons is in contrast to the visual system. Activity-dependent pruning is well established in the visual system where coordinated functional activity among contiguous retinal ganglion cells stabilizes elements of the axonal arbor while uncorrelated branches are lost or pruned (Stryker and Strickland, 1984; Sretavan and Shatz, 1986; Shatz and Stryker, 1988; Cline and Constantine-Paton, 1989). The visual system, therefore, uses coordinated activity among contiguous ganglion cells to dictate the specificity of central projeetions by stabilizing their axons within a narrowly defined area of neuropil. However, it is more difficult for the olfactory system to work in this manner. The olfactory epithelium provides four broad zones in which putative odor receptors are expressed. Within each zone there appears to be a random or stochastic distribution of receptors (Ressler et al., 1993; Vassar et al., 1993). Yet, within the OB a very specific topographical map is formed in which axons expressing a given receptor innervate only two or a small number of glomeruli (Mombaerts et al., 1996). Due to the broad distribution of ORNs in the epithelium that project to a single glomerulus, it could be difficult for the olfactory system to employ adjacent stabilization in formation of the glomerular map. Thus, one might predict that the olfactory system would employ measures other than activity-dependent mechanisms to guide the development and arborization of ORN axons. Moreover, given that ORNs appear rather broadly tuned to odors with extensive overlap

in their response spectra, it is difficult to envision how odor-induced activity could occur selectively in the small subset of ORNs projecting to a single glomerulus.

Mombaerts et al. (1996) also suggest that ORN development is unlikely to be based on pure activity-dependent mechanisms. Introduction of a *tau*-Lac-Z receptor downstream from specific odor receptors enabled the authors to visualize axons of ORNs expressing those receptors. It appears that the axons from ORNs expressing the same receptor converge onto only 1-3 glomeruli in the OB, an observation consistent with the prior reports of localization of mRNA to specific odor receptors to only a few OB glomeruli (Vassar et al., 1994; Ressler et al., 1994). Mombaerts et al. (1996) further suggest that early in development ORN axons converge onto relatively spatially fixed glomeruli, thus arguing against the notion of non-specific or exuberant projections. Oland and Tolbert (1996), as well, argued for a certain degree of activity independence in olfactory system development. They reported that in the moth *Manduca sexta* elimination of Na+ dependent activity with tetrodotoxin prior to ORN innervation of the antennal lobe had no effects on organization in the adult. Despite the absence of functional activity in the nerve, the mature brains showed no discernible changes in the fields occupied by receptor axons, in the distribution of glial cells, or in dendritic branching patterns of a 5-HT neuron. They concluded that glomerular formation in *Manduca sexta* occurs largely independent of primary afferent activity. Both of these studies are clearly consistent with our current finding that the morphometric features of developing ORN axons in the rat show no evidence of developmental exuberance or non-specific targeting.

Although our data argue against the notion of exuberant development of ORN axons there is evidence of early exuberance elsewhere in the olfactory pathway. Malun and Brunjes (1996) demonstrated an early multi-glomerular innervation by mitral cell apical dendrites that was pruned to a single glomerulus during maturation. In a similar vein, Greer (1984) demonstrated an overproduction of granule cell dendritic spines

around PND 12 in the rat that was subsequently reduced to adult levels by PND 21. Thus, while a broad and non-specific projection of ORN axons may not be a fundamental feature of development in the olfactory system, activity-dependent mechanisms could still contribute to the final synaptic organization of both projeetion and interneurons in the OB.

While the results from our data agree with how one might expect the olfactory system to behave, there were eertain assumptions made in gathering this data which must be addressed. At any given age both mature and immature axons were present since axonal innervation of the OB begins around E16 but continues throughout life. We hypothesized that the population of axons would be more heavily weighted towards immature axons in younger animals. Our data appear consistent with this hypothesis. If the population of axons studied was not age weighted through development, one would expect to have seen morphometric features remain fairly constant throughout age groups. Rather, variables we measured in this study showed the trend of an age dependent linear increase which plateaued at the adult values. Although the trend is evident (cf. Table 2), the MANOVA did not demonstrate statistically significant differences between the age groups. This is also most likely a reflection of the heterogeneous composition of axons at any given age. As noted above, ORN axons first begin to reach the OB around E16, however, new ORNs continue to be generated and extend axons to the OB throughout the postnatal period. Thus, while the postnatal ages we studied represented convenient developmental landmarks, it is likely that the age of the ORN axons was heterogeneous.

Another assumption was that a random selection of axons at any given age group would be impregnated by the Golgi methods employed. Indeed, historically Golgi methods have been shown to be a useful tool with which to impregnate randomly a population of axons. The results of this study support the non-selectivity of the Golgi impregnations. In particular, since the data revealed axons of both a simple and eomplex character at each of the ages studied, but with a trend towards increasing complexity in

older animals, it seems likely that the Golgi procedure did not selectively impregnate axons of a given or restricted age.

The 9 DPN set of axons is the only age group which seems to stray from the trend of an initial linear age dependent increase in morphometric values with a plateauing upon obtaining the adult value. Specifically, the morphometric values for 9 DPN axons are on average smaller than this trend would project. It is not clear as to why this occurred, however, it is interesting to note that when the 9 DPN set is corrected to glomerular area or diameter, the values obtained correlate well with values from other age groups. Regardless, with or without correction, the 9 DPN set of axons do not support hypertrophy of axonal arbors due to the fact that their morphometric values do not peak above the adult values.

The results of the adult morphometric parameters in this study (21 DPN) differ somewhat from those recorded by Halasz and Greer (1993). In particular the number of branch points for 21 DPN in this study (17.00 ± 1.98) versus those in the prior study ($6.84 \pm .51$) appeared notable. Given the otherwise stable nature of the quantitative features described in the current study, it seems that the most probable explanation for the differences between the two studies is inter-experimenter variability in criteria for selecting axons for reconstruction. In part, this seems the most plausible explanation because the same set of stained 21 DPN slides were employed in both studies. Moreover, it is paramount that we emphasize that despite some quantitative differences, the qualitative results for adult morphometric values are consistent between the current study and Halasz and Greer (1993). In particular, in both studies individual ORN axons arborized in a similar fashion within restricted glomerular subcompartments. In discussing differences between this and the prior study, it also of importance to emphasize that in the current study the criteria for reconstruction of axonal arbors was applied throughout all age groups in an equivalent fashion. However, as noted above the 21 DPN slides were generated several years prior to the 0, 3, 6, 9 and 12 DPN slides also

utilized in the current study. Thus the differences we observed in axonal morphometrics between age groups, which suggest a lack of axonal exuberance, are likely to be accurate and not a reflection of experimental variability.

By demonstrating a precise glomerular innervation by ORNs without evidence of axonal exuberance this study suggests that a set of molecular markers must underlie the guidance and targeting of ORN axons. One likely candidate for playing a part in the targeting of ORNs is the odor receptor itself (Singer et al., 1995), although recent research implies that it is likely not the only pathfinding moiety involved since replacement of the receptor does not completely redirect the axons to new targets (Mombaerts et al., 1996). Among other candidates that may influence ORN extension, Gong and Shipley (1996) demonstrated specific patterns of expression of NCAM and L1 by ORNs. In addition, laminin, a basement membrane glycoprotein involved in cell adhesions, is also present in the olfactory pathway and has been demonstrated to be a preferential substrate for ORN axon extension (Kafitz and Greer, 1997). Similarly, a variety of cell surface glycoproteins have been described for the olfactory pathway and subsets of glomeruli (Mori, 1987; Schwob and Gottleib, 1988; Schwarting et al., 1992) as well as the differential binding of lectins to subsets of ORNs and their axons (Key and Akeson, 1990, 1993; Treloar et al., 1996; Puche et al., 1996). The presence of specific molecular phenotypes on ORN axons, in conjunction with the results from the current study, argues for a high degree of specificity in the targeting of ORN axons to and within glomeruli. Thus, it seems reasonable to suggest that targeting by an ORN axon could depend upon permutations within a series of substrate markers as it travels from the olfactory epithelium to the OB, to a specific glomerulus within the OB, to a subcompartment within the glomerulus and finally to arborize to the final adult morphology.

In summary, our results suggest that ORN axons innervate specific glomeruli, arborize to their final adult pattern and stabilize without evidence of exuberance or non-

specific targeting to secondary glomeruli. Thus, activity-dependent mechanisms seem unlikely to play a significant role in establishing the projection of primary afferent ORN axons to the OB. Rather, the data suggest the presence of highly specific molecular maps/substrates that guide ORN axons to their correct glomerular and intraglomerular targets.

REFERENCES

Bayer, S.A., and J. Altman (1975) The effects of X-irradiation on the postnatally-forming granule cell populations in the olfactory bulb, hippocampus, and cerebellum of the rat. Exp. Neurol. *48*:167-174.

Cajal, R.S. (1911) Histologie du Systeme Neurneux de l'Hommes et des Vetebres. Tome 2. Institut Ramon y Cajal, Madrid.

Cameron, H.A., C.K. Kaliszewski, and C.A. Greer (1991) Organization of mitochondria in olfactory bulb granule cell dendritic spines. Synapse *7*:181-192.

Chess, A., I. Simon, H. Cedar, and R. Axel (1994) Allelic inactivation regulates olfactory receptor gene expression. Cell *78*:823-834.

Cline, H.T., and M. Constantine-Paton (1989) NMDA receptor antagonists disrupt the retinotectal topographic map. Neuron *3*:413-426.

Constantine-Paton, M., H.T. Cline, and E. Debski (1990) Patterned activity, synaptic convergence, and the NMDA receptor in developing visual pathways. Ann. Rev. of Neurosci. *13*:129-154.

Garrity, P.A., and S.L. Zipursky (1995) Neuronal target recognition. Cell. *83*:177-185.

Getchell, M.L., B. Zielinski, and T.V. Getchell. (1988) Odorant and autonomic regulation of secretion in the olfactory mucosa. In: Margolis, F.L., and T.V. Getchell, eds. *Molecular neurobiology of the olfactory system*. New York: Plenum Press. 71-98.

Golgi, C. (1875) Sulla Fina Structura dei Bulbi Olfactorii. Rome Reggio-Emilia.

Gong, Q., and M.T. Shipley (1995) Evidence that pioneer olfactory axons regulate telencephalon cell cycle kinetics to induce the formation of the olfactory bulb. Neuron *14*:91-101.

Gong, Q., and M.T. Shipley (1996) Expression of extracellular matrix molecules and cell surface molecules in the olfactory nerve pathway during early development. J. Comp. Neurol. *366*:1-14.

Goodman, C.S., and C.J. Shatz (1993) Developmental Mechanisms that generate precise patterns of neuronal connectivity. Cell. *72*:77-98.

Greer, C.A., W.B. Stewart, M.H. Teicher, and G.M. Shepherd (1982) Functional development of the olfactory bulb and a unique glomerular complex in the neonatal rat. J. Neurosci. *2*:1744-1759.

Greer, C.A. (1984) A golgi analysis of granule cell development in the neonatal rat olfactory bulb. Soc. Neurosci. Abst. *10*:531.

Greer, C.A. (1991) Structural Organization of the Olfactory System. In: Getchell, T.V., et al., eds. *Smell and Taste in Health and Disease*. New York: Raven Press. 65-81.

Greer, C.A., J.C. Bartolomei, and J.M. Dembner (1994) Organization of primary afferent and local circuit synapses in the olfactory glomerulus. Microscopy Soc. America Proc. *52:*148-149.

Greer, C.A., H. Kim, and K. Chiu (1995) Subcompartmental organization of the rat olfactory bulb glomerulus. Soc. Neurosci. Abs. *21:*1184.

Guthrie, K.M., A.J. Anderson, M. Leon, and C.M. Gall (1993) Odor-induced increases in *c-fos* mRNA expression reveal an anatomical unit for odor processing in olfactory bulb. Proc. Nat. Acad. Sci., USA *90*: 3329-3333.

Halasz, N., and C.A. Greer (1993) Terminal arborizations of olfactory nerve fibers in the glomeruli of the olfactory bulb. J. Comp. Neurol. *337*:307-316.

Kafitz, K.W., and C.A. Greer (1997) The role of laminin in axonal extension from olfactory receptor cells. J. Neurobiol. *32*:298-310.

Key, B., and R.A. Akeson (1990) Olfactory neurons express a unique glycosylate form of the neural cell adhesion molecule (N-CAM). J. Cell Biol. *110*:1729-1743.

Key, B., and R.A. Akeson (1993) Distinct subsets of sensory olfactory neurons in mouse: possible role in the formation of the mosaic olfactory projection. J. Comp. Neurol. *335*:355-368.

Laing, D.G., R.L. Doty, and W. Breipohl, eds. (1991) *The Human Sense of Smell*. New York: Springer-Verlag. 8-20.

Land, L.J., R.P. Eager, and G.M. Sheperd (1970) Olfactory nerve projections to the olfactory bulb in rabbit: demonstration by means of a simplified ammoniacal silver degeneration technique. Brain Res. *23*:250-254.

Malmun, D., and Brunjes, P.C. (1996) Development of olfactory glomeruli: temporal and spatial interactions between olfactory receptor axons and mitral cells in opossums and rats. J. Comp. Neurol. *368:*1-16.

Menco, B., and A.L. Farbman (1985) Genesis of cilia and microvilli of rat nasal epithelia during prenatal development. I. J. Cell Sci. *78*:283-310.

Menco, B., and A.L. Farbman (1985) Genesis of cilia and microvilli of rat nasal epithelia during prenatal development. II. J. Cell Sci. *78*:283-310.

Mombaerts, P., F. Wang, C. Dulac, S.K. Chao, A. Nemes, M. Mendelsohn, J. Edmondson, and R. Axel (1996) Visualizing an olfactory sensory map. Cell *87*: 675-686.

Monti-Graziadei, G.A., R.S. Stanley, and P.P.C. Graziadei (1980) The olfactory marker protein in the olfactory system of the mouse during development. Neurosci. *5*:1239-1252.

Mori, K. (1987) Monoclonal antibodies (2C5 and 4C9) against lactoseries carbohydrates identify subsets of olfactory and vomeronasal receptor cells and their axons in the rabbit. Brain Res. *408*:215-221.

Oland, L. A., and L.P. Tolbert (1996) Multiple factors shape development of olfactory glomeruli: insights from an insect model system. J. Neurobiol. *30:* 92-109.

Onoda, N. (1992) Odor-induced fos-like immunoreactivity in the rat olfactory bulb. Neurosci. Letters *137*: 157-160.

Puche, A.C., F. Poirier, M. Hair, P.F. Bartlett, and B. Key (1996) Role of galectin-1 in the developing mouse olfactory system. Develop. Biol. *179*:274-287.

Ressler, K.J., S.L. Sullivan, and L.B. Buck (1993) A zonal organization of odorant receptor gene expression in the olfactory epithelium. Cell *73*:597-609.

Ressler, K.J., S.L. Sullivan, and L.B. Buck (1994) Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. Cell *79*:1245-1255.

Sallaz, M., and F. Jourdan (1993) C-*fos* expression and 2-deoxyglucose uptake in the olfactory bulb of odour-stimulated awake rats. NeuroReport *4*:55-58

Santacana, M., M. Heredia, and F. Valverde (1992) Transient pattern of exuberant projections of olfactory axons during development in the rat. Develop. Brain Res. *70*:213-222.

Schwarting, G.A., G. Deutsch, D.M. Gattey, and J.E. Crandall (1992) Glycoconjugates are stage- and position-specific cell surface molecules in the developing olfactory system, 2: unique carbohydrate antigens are topographic markers for selective projection patterns of olfactory axons. J. Neurobiol. *23*:130-142.

Schwob, J.E., and D.I. Gottlieb (1988) Purification and characterization of an antigen that is spatially segregated in the primary olfactory projection. J. Neurosci. *8*:3470-3480.

Serby, M.J., and K.L. Chobor, eds. (1992) *Science of Olfaction*. New York: Springer-Verlag. 31-37.

Sharp, F.R., J.S. Kauer, and G.M. Sheperd (1975) Local sites of activity-related glucose metabolism in rat olfactory during olfactory stimulation. Brain Res. *98*:596-600.

Shatz, C.J., and M.P. Stryker (1988) Prenatal tetrodotoxin infusion blocks segregation of retinogeniculate afferents. Sci. *242*:87-89.

Shatz, C.J. (1990) Impulse activity and the patterning of connections during CNS development. Neuron. *5*: 745-756.

Shepherd G.M. (1989) Studies of development and plasticity in the olfactory sensory neuron. J. Physiol, Paris. *83*:240-5.

Shepherd, G.M., and C.A. Greer (1998) Olfactory Bulb. In: Shepherd, G.M., ed. *The Synaptic Organization of the Brain*. New York: Oxford University Press. 159-203.

Singer, M., G.M. Shepherd, and C.A. Greer (1995) Olfactory receptors guide axons. Nature *377*:19-20.

Sretavan, D. W., and Shatz, C. J. (1986). Prenatal development of retinal ganglion cell axons: segregation into eye-specific layers. J. Neurosci. *6*: 234-251.

Stewart, W.B., J.S. Kauer, and G.M. Shepherd (1979) Functional organization of rat olfactory bulb analyzed by the 2-deoxyglucose method. J. Comp. Neurol. *185*:715-734.

Strotmann, J., A. Beck, S. Kubick, and H. Breer (1995) Topographic patterns of odorant receptor expression in mammals: a comparative study. J. Comp. Physiol. A *177*:659-666.

Stryker, M. P., and Strickland, S. L. (1984). Physiological segregation of ocular dominance columns depends on the pattern of afferent electrical activity. Invest. Ophthalmol. Vis. Sci. (suppl.) *25*: 278.

Treloar, H., E. Walters, F. Margolis, and B. Key (1996) Olfactory glomeruli are innervated by more than one distinct subset of primary sensory olfactory neurons in mice. J. Comp. Neurol. *367*:550-562.

Valverde, F., M. Santacana, and M. Heredia (1992) Formation of an olfactory glomerulus: morphological aspects of development and organization. Neurosci. *49*:255-275.

Vassar, R., J. Ngai, and R. Axel (1993) Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. Cell *74*:309-318.

Vassar, R., S.K. Chao, R. Sitcheran, J.M. Nunez, L.B. Vosshall, and R. Axel (1994) Topographic organization of sensory projections to the olfactory bulb. Cell *79*:981-991.

HARVEY CUSHING / JOHN HAY WHITNEY
MEDICAL LIBRARY

MANUSCRIPT THESES

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS DATE

YALE MEDICAL LIBRARY
3 9002 01048 7636